

Antimicrobial Activity of Metronidazole in Anaerobic Bacteria

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The antimicrobial activity of metronidazole was investigated in anaerobic bacteria by use of time-viability studies. This antimicrobial agent has a rapid onset of bactericidal activity under proper reducing conditions. The bactericidal rates were not affected by inoculum size or nutritional requirements, nor by inhibition of growth and protein synthesis by chloramphenicol. Using supernatant fractions of actively growing cultures of susceptible organisms, we observed a disappearance of metronidazole and a loss of biological activity, but there was no significant change in preparations from resistant bacteria. The decrease in drug concentration with susceptible cells occurred during the time that its bactericidal action was being exerted. Extracts from susceptible organisms rapidly reduced the concentration of metronidazole, confirming previous observations which suggest that the drug acts as a terminal electron acceptor. Radioisotope experiments with [^{14}C]metronidazole revealed that the compound was taken up by both resistant and susceptible bacteria, although there was a difference in rate and extent of accumulation. These studies demonstrate that metronidazole's antimicrobial activity against anaerobic bacteria is bactericidal and independent of growth rate, and that it involves the uptake and metabolism of the compound.

Metronidazole was first introduced into clinical medicine in 1959 for the treatment of *Trichomonas vaginalis* infections (2), and it has been subsequently used for invasive amebiasis and giardiasis (4, 22). Recent studies have documented its in vitro bactericidal activity against anaerobic bacteria (14). Metronidazole appears to be efficacious in clinical anaerobic infections (18), and two groups have reported its use prophylactically in gynecological and colonic surgery (6, 21). The common denominator in these diverse indications is this antimicrobial agent's exclusive activity against anaerobic microorganisms.

The majority of published reports on the mechanism of action of metronidazole have concentrated on the effects in protozoa and tissue culture cells (3, 8, 19). Our laboratory became interested in the drug because of its activity against clinically significant anaerobic bacteria. In addition, we thought that the rapid growth and simple culture requirements of anaerobic bacteria would make them excellent models for the investigation of metronidazole's antimicrobial action.

Preliminary studies of the activity of metronidazole in anaerobic bacteria have been carried out, but the results have been somewhat controversial. Ralph and Kirby suggested that metro-

nidazole has a marked bactericidal effect which occurs after a prolonged lag phase (16). There remains the possibility, however, that this prolonged lag period was an artifact of methodology. The purpose of our study was to investigate the antimicrobial activity of metronidazole on anaerobic bacteria, utilizing optimal anaerobic conditions, and to examine its uptake and disappearance in these microorganisms.

MATERIALS AND METHODS

Organisms. The anaerobic bacteria utilized in this study were isolated from clinical material processed in our laboratory and were identified according to the criteria of the Virginia Polytechnic Institute *Anaerobic Manual* (7). In addition, two *Bacteroides fragilis* isolates (K-1 and K-14) were obtained from William Kirby, Seattle, Wash. Minimal inhibitory concentrations (MICs) of metronidazole for these isolates were determined by previously described techniques (17).

Chemicals. Metronidazole standard compound and [^{14}C]metronidazole (SC-10295, lot Z11-206, specific activity 17.55 $\mu\text{Ci}/\text{mg}$) was obtained from Searle Laboratories, Chicago, Ill.

Media. All cultures were maintained on prereduced supplemental brain heart infusion agar slants (Scott Laboratories, Fiskeville, R.I.). Plate medium used for purification was brucella agar with 5% defibrinated sheep blood and 10 μg of vitamin K_1 per ml. The broth medium was prereduced brain heart infusion

supplemented with 0.1 μg of vitamin K₁ per ml and 0.05% hemin (BHIS).

Viability studies. Standard killing curves were performed in an anaerobic chamber with prereduced anaerobic sterilized (PRAS) techniques (1, 7). The chamber contained an atmosphere of 85% N₂, 10% H₂, and 5% CO₂. The inoculum was grown overnight to yield approximately 10⁸ colony-forming units (CFU)/ml, subcultured to fresh broth, grown to mid-log phase, and diluted to give approximately 10⁶ to 10⁷ CFU/ml. Metronidazole was then added to give a final concentration of 10 μg /ml. The control and test broths were incubated at 35°C in the chamber. Colony counts were performed at 0, 1, 2, 4, and 24 h by the drop method, and the plates were counted after 24 h of incubation. CFU per milliliter were plotted against time to determine the rate of killing. The drug was considered bactericidal if there was a decrease of 3 logs or more in the CFU per milliliter.

Effect of inoculum size. The effect of increasing inoculum on the bactericidal activity of metronidazole was determined by utilizing standard killing curves, described above, with inocula of 10³ to 10⁴, 10⁵ to 10⁶, and 10⁶ to 10⁷ CFU/ml, in 10 μg of metronidazole per ml. The counts were plotted to log₁₀ versus time, and the rate of killing was calculated from the slope of the curve by the formula $\Delta(N_0, N_2)/\Delta(t_0, t_2)$ from 0 to 1 h and $\Delta(N_0, N_2)/\Delta(t_0, t_2)$ from 0 to 2 h, with the results tabulated as log decrease per hour. The effect of inoculum size on the MIC of metronidazole was tested with broth dilution methods, as previously described, modified for use in the anaerobic chamber.

Effect of nutritional requirements. The nutritional requirements for the antimicrobial action of metronidazole versus *B. fragilis* were examined by comparing killing curves in minimal and complex medium. The minimal, defined medium, prepared by the method of Varel and Bryant (20), contained glucose as the carbon source, minerals, FeSO₄, (NH₄)₂SO₄ as the nitrogen source, vitamin B₁₂, and hemin. This medium was prepared fresh for each experiment. The complex medium was BHIS, described above.

Activity on chloramphenicol-inhibited cells. The antimicrobial activity of metronidazole was studied in cells of *B. fragilis* inhibited by chloramphenicol to determine the effect of reduced protein synthesis on the bactericidal rate (5). Cultures of *B. fragilis* were grown to early log phase in 200 ml of BHIS, and then each was equally divided into two tubes. Chloramphenicol was added to one of the tubes to give a final concentration of 8 μg /ml. After 2 additional h of incubation, the cultures were further divided into 50-ml portions, and metronidazole (10 μg /ml) was added to one of the control cultures and to one of the cultures containing chloramphenicol. Colony counts were obtained at 0, 2, 3, 4, 5, 6, and 24 h.

Assay for alteration of metronidazole. The resistant organism *Escherichia coli* 10 and the susceptible strain *B. fragilis* 8 were grown in minimal medium to early log phase, and 8 μg of metronidazole per ml was added to each culture flask. Control cultures in minimal medium were maintained under identical conditions. At 0, 5, 10, 20, 30, and 60 min, 5-ml samples were removed and centrifuged at 27,000 $\times g$ for 10 min; the supernatant fluids were removed and held at

4°C. After completion of the sampling, the concentration of drug in the medium supernatant fluid was determined spectrophotometrically at 320 and 340 nm (15).

Metabolism of metronidazole by bacterial extracts. Bacterial extracts were prepared from the metronidazole-susceptible organisms *B. fragilis* 8 and *Clostridium perfringens* 41 and from the resistant organisms *E. coli* 10 and *Propionibacterium acnes* 121. The organisms were grown in 100 ml of BHIS into late log phase (approximately 10⁸ CFU/ml) and were harvested under anaerobic conditions by centrifugation at 15,000 rpm for 20 min, with the use of plastic bottles with butyl rubber gaskets. The pellet was washed in the anaerobic chamber with 0.02 M dithiothreitol at pH 7 and was sonically treated. The sonic extract was reduced in the anaerobic chamber for 2 h. The assay mixture contained 0.1 μmol of co-enzyme A, 60 μmol of pyruvate, 300 μmol of metronidazole, 0.5 μmol of methyl viologen, and 0.4 ml of bacterial extract in a total volume of 3.8 ml. Control tubes containing various ingredients of the above mixture were included. All manipulations were carried out in the anaerobic chamber; test mixture and controls were incubated at 24°C for 2 h. The reaction mixture was centrifuged to remove cellular debris, and the supernatant fluid was used for spectrophotometric determination of the drug concentrations. Biological activity of the metronidazole in the supernatant fluid was determined by the agar well microbiological assay (9). Protein concentrations were determined by the method of Lowry et al. (11).

[¹⁴C]metronidazole uptake. The uptake of [¹⁴C]-metronidazole was studied in *B. fragilis* and *E. coli* by use of PRAS (7) and anaerobic chamber techniques (1). The location of the ¹⁴C radioactive tag in the hydroxyethyl side chain is shown in Fig. 1. Pure cultures of *B. fragilis* and *E. coli* were separately inoculated into 100 ml of BHIS with cysteine (BHIS-C), incubated for 5 h, and then dispensed into 5-ml glass tubes with butyl rubber stoppers and removed from the chamber. [¹⁴C]metronidazole, diluted 10-fold with carrier, was added to each tube under CO₂ gas to give a final concentration of 10 μg /ml. At various intervals over 4 h, duplicate 50- μl samples were removed, and the cells were collected on 0.45- μm membrane filters (Millipore Corp.) which had previously been washed with 5 ml of BHIS-C broth containing 10 μg of unlabeled metronidazole per ml. The cells were washed with 2 ml of the BHIS-C, placed in vials containing 10 ml of Aquasol, (New England Nuclear, Boston, Mass.) and counted in a Beckman LS 230 liquid scintillation counter. A control 50- μl sample of 10 μg of [¹⁴C]metronidazole per ml of BHIS-C broth contained 3,734 counts. (Filters did not retain counts from the control ¹⁴C-labeled broth after a pretreatment with 5 ml of unlabeled metronidazole in BHIS-C followed by a wash with 2 ml of BHIS-C.)

RESULTS

Antimicrobial activity. Agar dilution susceptibility testing revealed that the 13 anaerobic bacteria included in this investigation were in-

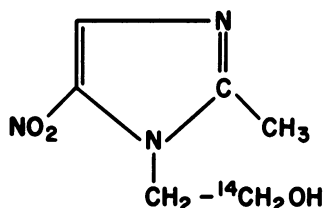
FIG. 1. Structure of [^{14}C]metronidazole.

TABLE 1. Minimal inhibitory concentration (MIC) of metronidazole for the anaerobic and aerobic bacteria tested

Isolate	MIC ($\mu\text{g/ml}$)
<i>Bacteroides fragilis</i> 8 ^a	1.0
<i>B. fragilis</i> 441	2.0
<i>B. fragilis</i> 563	1.0
<i>B. fragilis</i> K-1	1.0
<i>B. fragilis</i> K-14	1.0
<i>B. ovatus</i> 464	0.5
<i>B. ovatus</i> 496	0.5
<i>B. distasonis</i> 375	4.0
<i>B. distasonis</i> 483	2.0
<i>B. distasonis</i> 485	2.0
<i>Bacteroides</i> sp. 507	0.5
<i>Clostridium perfringens</i> 41	0.5
<i>C. perfringens</i> 249	4.0
<i>C. perfringens</i> 261	8.0
<i>C. bifermentans</i> 120	2.0
<i>Propionibacterium acnes</i> 121	>500
<i>Escherichia coli</i> 10	>500

^a Tufts Anaerobic Laboratory number.

hibited by 8 μg or less of metronidazole per ml (Table 1).

The drug had a prompt onset of bactericidal activity against the *Bacteroidaceae* (Fig. 2). At 4 h after addition of the drug, there was a greater than 5-log decrease with seven isolates and 3- to 4-log decreases with the others. At 24 h, there was no growth with seven isolates, and there were fewer than 10^3 CFU/ml with three isolates. The two strains from Seattle, previously studied by Ralph and Kirby (16), were promptly killed with our techniques.

The bactericidal activity of metronidazole against clostridia was similar to that against *Bacteroidaceae* (Fig. 3). There was a rapid onset of killing, with the colony counts decreasing 3 to 4 logs in 1 h.

Inoculum size of two strains of *B. fragilis* had no significant effect on metronidazole's bactericidal rate (Table 2).

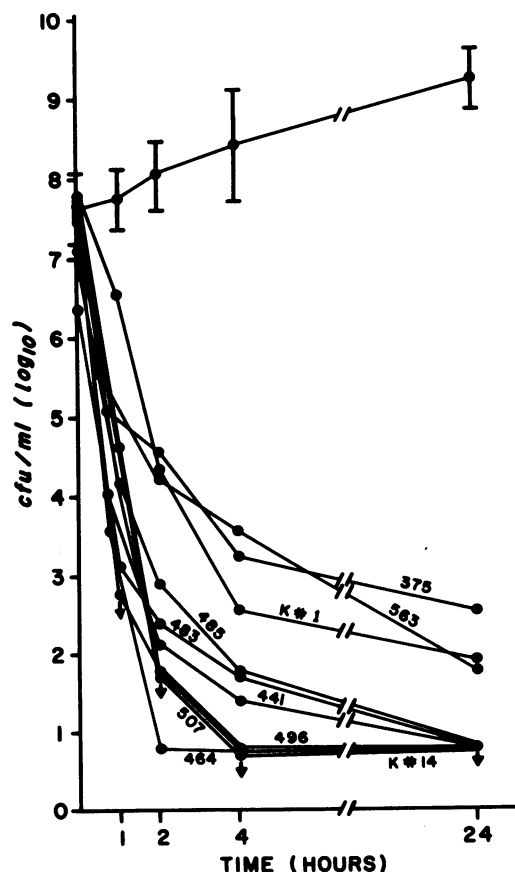
There were no significant differences in the bactericidal rates against three isolates of *B. fragilis* grown in minimal versus enriched media. Figure 4 illustrates the rapid decrease in CFU of *B. fragilis* 464 grown in both media.

The inhibition of growth and protein synthesis

by chloramphenicol had no effect on the bactericidal activity of metronidazole against *B. fragilis* (Fig. 5). The organism was exposed to chloramphenicol for 2 h, and after the addition of metronidazole there was prompt bactericidal activity with a decrease of greater than 6 logs in both the rapidly growing and the chloramphenicol-inhibited cells.

Effect of actively growing cells on the concentration of metronidazole. With a resistant strain of *E. coli*, the concentration of metronidazole decreased only 4 to 5% over a 60-min period (Fig. 6). However, with the susceptible *B. fragilis* strain, there was a greater than 90% decrease in the amount of drug measured spectrophotometrically. Concomitantly, there was a loss of biologically active drug in the medium from the *B. fragilis* culture as determined by the agar well diffusion assay, whereas there was no significant loss of bioactive drug in the *E. coli* culture.

Decrease in concentration of metronidazole by bacterial extracts. Metronidazole was

FIG. 2. Viability of 10 *Bacteroidaceae* organisms treated with metronidazole.

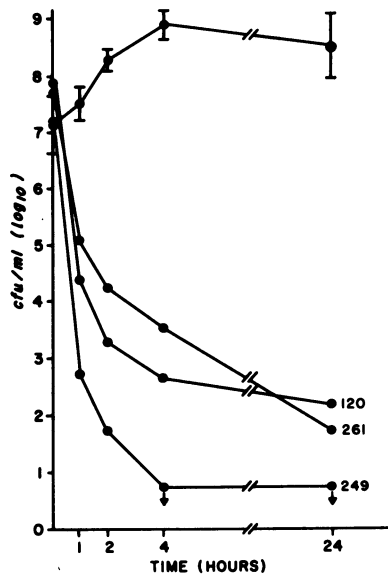


FIG. 3. Viability of three clostridia treated with metronidazole.

TABLE 2. Effect of inoculum size on the bactericidal rate of metronidazole against two isolates of *B. fragilis*

Strain	Inoculum size	Decrease in CFU (log ₁₀)	
		1 h	2 h
K-1	10 ⁷ -10 ⁸	2.63	3.81
	10 ⁶ -10 ⁶	2.82	4.78
	10 ³	>2.29	
563	10 ⁸	1.97	3.75
	10 ⁶ -10 ⁶	2.02	2.75
	10 ³	>1.40	>3.18

incubated under anaerobic conditions with sonic extracts prepared from the susceptible organisms *B. fragilis* and *C. perfringens*, and there was a rapid reduction in the concentration of the drug (15 and 25 $\mu\text{g/h}$ per mg of protein, respectively). However, there was no loss of the compound when it was exposed to sonic extracts from the resistant strains *P. acnes* and *E. coli*. This loss of metronidazole activity with susceptible organisms was not observed with supernatant fluid from the sonic extracts nor when the sonic extracts were incubated in atmospheric oxygen.

In tubes containing both methyl viologen and metronidazole, several minutes were required for sonic extracts of susceptible organisms to reduce the dye, indicated by formation of a blue color. Extracts incubated with methyl viologen in the absence of metronidazole displayed a rapid formation of the blue color. When metro-

nidazole was introduced in excess to the methyl viologen, the dye was not reduced until all of the metronidazole had disappeared. These observations indicate that methyl viologen acts as an

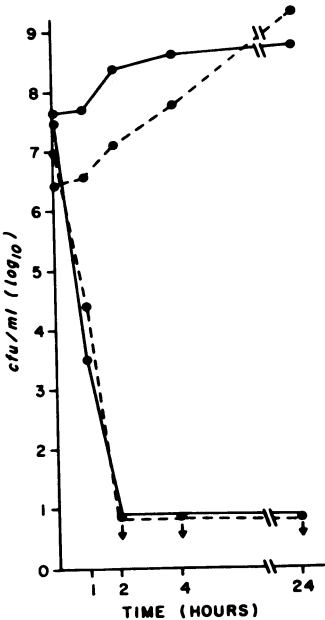


FIG. 4. Effect of medium on the viability of *B. fragilis* 8 grown with metronidazole. (-----) Minimal medium; (—) enriched medium.

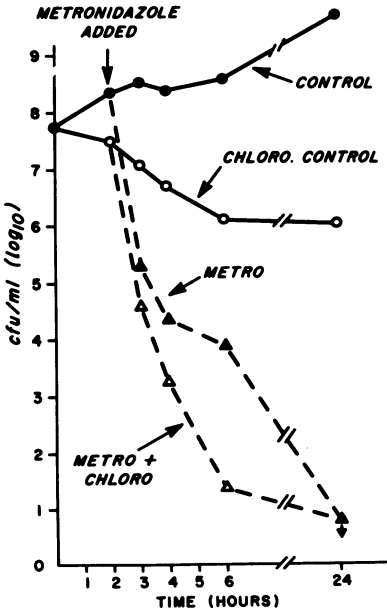


FIG. 5. Effect of chloramphenicol on the viability of *B. fragilis* 8 treated with metronidazole.

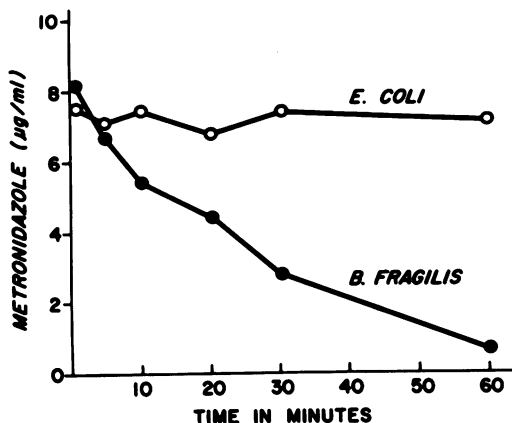


FIG. 6. Concentration of metronidazole in the medium of a resistant *E. coli* (○) and a susceptible *B. fragilis* (●) strain.

intermediate electron carrier and metronidazole is the terminal electron acceptor.

[¹⁴C]metronidazole uptake. There was uptake of metronidazole by both the resistant *E. coli* and the susceptible *B. fragilis* grown under anaerobic conditions, although there were differences in the rate and extent (Fig. 7). A rapid uptake of labeled drug (28%) by *B. fragilis* occurred during the first 2 h, during which time metronidazole is known to exert its bactericidal action. On the other hand, only 8% of the isotope was taken up by *E. coli* in 2 h.

DISCUSSION

The results of this study demonstrate that, under the proper reducing conditions, metronidazole has a rapid onset of bactericidal activity against susceptible anaerobic bacteria. These findings are divergent from those of Ralph and Kirby (16), who showed a prolonged lag phase followed by a rapid decrease in viable cells. These investigators used anaerobic jar techniques which exposed the medium and organism to atmospheric oxygen, thus raising the oxidation reduction potential (Eh). The establishment of anaerobiosis and reduction of Eh takes several hours in these jars (W. Seip, G. Evans, and J. Abramson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, C89, p. 40). In our studies, the Eh was already reduced in the anaerobic chamber, and the bactericidal activity of metronidazole was immediately evident. Mueller and Lindmark have demonstrated that a low Eh is essential for the antimicrobial activity of this compound (13). The prolonged lag period probably reflects vagaries of Eh associated with anaerobic jars.

We examined other factors which might affect the in vitro activity of metronidazole. Neither

the inoculum size nor the growth rate of the organisms altered the bactericidal activity of the drug. Although increasing the inoculum size from 10^3 to 10^8 cells did not change rate of killing, a longer time was required to kill the entire population when the inoculum was high. These findings are in contrast to those observed with other bactericidal agents such as the penicillins and cephalosporins (12).

Different generation times of an organism had little effect on the activity of metronidazole. When grown in minimal medium, the generation time is prolonged because the organisms are required to synthesize macromolecules from simple element sources. The rate of killing was unchanged, however, when the organism was exposed to metronidazole in minimal or complex media.

Chloramphenicol-inhibited cells were used to test further the effect of growth rate on the activity of metronidazole. The prompt bactericidal action of metronidazole was still evident 2 h after the addition of chloramphenicol, when all protein synthesis should have been inhibited (5).

It is apparent that the activity of metronidazole is not affected by inoculum size, growth rate, or inhibition of protein synthesis. These findings may be explained by postulating that the antimicrobial activity of metronidazole is related to toxic intermediates produced during reduction of this compound (8). The antimicrobial effect may be caused by interaction of these intermediates with deoxyribonucleic acid, and there is evidence that radioactive products of metronidazole bind deoxyribonucleic acid in protozoa (8).

Lindmark and Mueller have recently reviewed

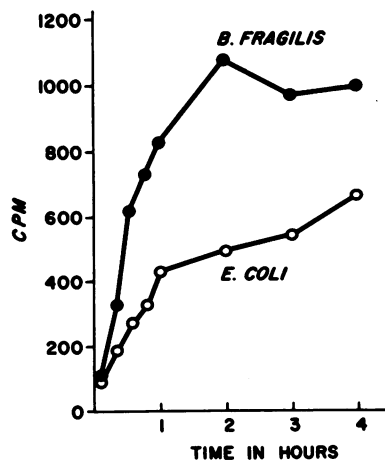


FIG. 7. Uptake of [¹⁴C]metronidazole by resistant (○) and susceptible (●) organisms.

the experimental data on the mechanisms of action of metronidazole (10). They have shown that the compound is taken up by susceptible anaerobic protozoa, a process which is oxygen sensitive (13). Furthermore, they have demonstrated that the reduction of the nitro group on the imidazole ring is related to the compound's antimicrobial activity against *Trichomonas* and *Clostridium*. Our preliminary results indicate that the drug is taken up by both susceptible and resistant bacteria, but only the susceptible organisms seem capable of metabolizing the drug. This suggests that the biochemical events observed in protozoa may also be responsible for the activity against susceptible anaerobic bacteria. Further investigations of the mechanism of action of metronidazole may be facilitated by utilizing *B. fragilis* because of its simple growth requirements and its adaptability to methods for the study of macromolecular synthesis.

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